Dynamic association between α-actinin and β-integrin regulates contraction of canine tracheal smooth muscle

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The adhesion junctions of smooth muscle cells may be dynamically regulated during smooth muscle contraction, and this dynamic regulation may be important for the development of active tension. In the present study, the role of α-actinin during smooth muscle contraction was evaluated in tracheal smooth muscle tissues and freshly dissociated cells. Stimulation with acetylcholine (ACh) increased the localization of α-actinin at the membrane of freshly dissociated smooth muscle cells, and increased the amount of β_1 integrin that coprecipitated with α-actinin from muscle tissue homogenates. GFP-α-actinin fusion proteins were expressed in muscle tissues and visualized in live freshly dissociated cells. GFP-α-actinin translocated to the membrane within seconds of stimulation of the cells with ACh. Expression of the integrin-binding rod domain of α-actinin in smooth muscle tissues depressed active contraction in response to ACh. Expression of the α-actinin rod domain also inhibited the translocation of endogenous α-actinin to the membrane, and inhibited the association of endogenous α-actinin with β_1-integrin in α-actinin immunoprecipitates from tissue extracts. However, the expression of α-actinin rod domain peptides did not inhibit increases in myosin light chain phosphorylation or actin polymerization in response to stimulation with ACh. Results suggest that contractile stimulation of smooth muscle causes the rapid recruitment of α-actinin to β-integrin complexes at the membrane, and that the recruitment of α-actinin to integrin complexes is necessary for active tension development in smooth muscle.

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Smooth muscle lines hollow organs that undergo large changes in shape and volume under physiological conditions *in vivo*, and the muscle must rapidly adapt its compliance and contractility to accommodate to changes in external mechanical forces. In differentiated smooth muscle cells and tissues, actin filaments attach to the cell membrane at adhesion sites, which are the sites of tension transmission between the contractile apparatus and the extracellular matrix (Small, 1985). At these sites, the extracellular domains of transmembrane integrins bind to extracellular matrix proteins, and the cytoplasmic domains of integrin proteins associate with cytoskeletal proteins that bind to actin filaments, thereby providing a route for the transmission of mechanical force between the contractile apparatus and the extracellular matrix (Wang *et al.* 1993; Burridge & Chrzanowska-Wodnicka, 1996; Yamada & Geiger, 1997; Critchley *et al.* 1999). Modulation of the connections of actin filaments to the cell membrane at these sites may provide a means for regulating cytoskeletal organization and tension development during the contractile activation of smooth muscle (Gunst *et al.* 1995, 2003; Gunst & Fredberg, 2003; Opazo Saez *et al.* 2004).

α-actinin is an actin-binding protein that also binds to integrin proteins, and can thereby serve as a link for the transmission of tension between the cytoskeleton and the extracellular matrix. The central rod domain of α-actinin, which consists of four spectrin-like repeats, binds to the cytoplasmic tail of β-integrins; while the N-terminal globular domain binds to F-actin (Baron *et al.* 1987; Mimura & Asano, 1987; Otey *et al.* 1990). In the focal adhesions of cultured fibroblasts, α-actinin is essential for the link between integrins and actin filaments and provides structural stability to adhesion sites (Greenwood *et al.* 2000; Rajfur *et al.* 2002; von Wichert *et al.* 2003; Corgan *et al.* 2004). α-actinin is also present at the membrane-associated dense plaques of smooth muscle, where it may serve an analogous role (Geiger *et al.* 1981; Fay *et al.* 1983; Small, 1985). In fibroblasts, α-actinin is recruited to sites of integrin clustering during cell adhesion (Edlund *et al.* 2001; von Wichert *et al.* 2003). A coordinated stepwise maturation of focal adhesions has been described,

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in which the recruitment of α-actinin is a later event in the maturation of small focal complexes (Laukaitis *et al.* 2001). The incorporation of α-actinin into these complexes is correlated with a force-dependent strengthening of integrin–cytoskeletal linkages (von Wichert *et al.* 2003). These studies suggest an important role for α-actinin in strengthening cytoskeletal linkages in cultured cells during cell migration and substrate adherence; however, it is not known whether regulation of the formation of integrin–cytoskeletal linkages is of functional importance in intact tissues or differentiated cells exposed to physiological stimuli.

In the present study, we investigated the possibility that the incorporation of α-actinin into integrin–cytoskeletal linkages might be a regulated event during the development of active tension in differentiated smooth muscle cells and tissues. We expressed green fluorescent protein (GFP)-fusion proteins for α-actinin in smooth muscle tissues and monitored their localization in living freshly dissociated smooth muscle cells. Stimulation with acetylcholine (ACh) initiated recruitment of α-actinin to the cell membrane within seconds. In smooth muscle tissues, the association of α-actinin with β-integrin protein complexes increased in response to contractile stimulation.

We evaluated the role of α-actinin in mediating connections between integrins and F-actin during tension development by expressing the integrin-binding rod domain of α-actinin in smooth muscle. The α-actinin rod domain contains binding sites for the cytoplasmic tail of β-integrin but it does not contain an actin-binding site (Otey *et al.* 1990; Kelly & Taylor, 2005). Expression of the α-actinin rod domain prevented the redistribution of endogenous α-actinin to the membrane in response to ACh in freshly dissociated smooth muscle cells. In intact muscle tissues, the α-actinin rod domain inhibited the association of α-actinin with β-integrins and depressed active tension development. We conclude that the recruitment of α-actinin the adhesion complexes of smooth muscle is regulated by contractile stimulation, and that the rapid recruitment of α-actinin to integrin–cytoskeletal connections is a necessary step in the development of active tension in smooth muscle.

Methods

Reagents and antibodies

β_1-integrin (Clone 18) antibody was obtained from Transduction Laboratories. α-actinin (MAb, clone BM75.2), actin (MAb, clone AC40), and talin (MAb clone 8D4) antibodies were from Sigma Chemical Co. Polyclonal vinculin and polyclonal myosin light chain antibodies were custom made by BABCO, Richmond, CA. Polyclonal and monoclonal (JL-8) GFP antibodies and monoclonal paxillin antibody (clone 349) were obtained from BD Biosciences, Palo Alto, CA. Alexa Fluor 488 and Alexa Fluor 546 were obtained from Molecular Probes Co. All other reagents were purchased from Sigma Chemical Co.

Preparation of smooth muscle tissues and measurement of force

Cross-breed dogs (20–25 kg) were anaesthetized with pentobarbital sodium (30 mg kg^{-1}, I.V.) and quickly exsanguinated in accordance with the guidelines of the Institutional Animal Care and Use Committee, Indiana University School of Medicine. A segment of the trachea was immediately removed and immersed in physiological saline solution (PSS) (mM: 110 NaCl, 3.4 KCl, 2.4 $CaCl_2$, 0.8 $MgSO_4$, 25.8 $NaHCO_3$, 1.2 KH_2PO_4, and 5.6 glucose). Smooth muscle strips (1.0 × 0.2–0.5 × 15 mm) were dissected free of connective and epithelial tissues. For the measurement of contractile force, muscle tissues were attached to force transducers and maintained within a tissue bath in PSS at 37°C.

Transfection of smooth muscle tissues with plasmids

Plasmids were introduced into tracheal smooth muscle strips by the method of reversible permeabilization (Tang *et al.* 2003; Opazo Saez *et al.* 2004; Zhang *et al.* 2005). After determination of the length of maximal isometric force (L_o), muscle strips were attached to metal mounts at L_o. The strips were incubated successively in each of the following solutions: Solution 1 (at 4°C for 120 min) containing (mM): 10 EGTA, 5 Na_2ATP, 120 KCl, 2 $MgCl_2$, and 20 N-tris (hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES); Solution 2 (at 4°C overnight) containing (mM): 0.1 EGTA, 5 Na_2ATP, 120 KCl, 2 $MgCl_2$, 20 TES, and 10 μg ml^{-1} plasmids; Solution 3 (at 4°C for 30 min) containing (mM): 0.1 EGTA, 5 Na_2ATP, 120 KCl, 10 $MgCl_2$, 20 TES; and Solution 4 (at 22°C for 60 min) containing (mM): 110 NaCl, 3.4 KCl, 0.8 $MgSO_4$, 25.8 $NaHCO_3$, 1.2 KH_2PO_4, and 5.6 dextrose. Solutions 1–3 were maintained at pH 7.1 and aerated with 100% O_2. Solution 4 was maintained at pH 7.4 and aerated with 95%O_2–5%CO_2. After 30 min in Solution 4, $CaCl_2$ was added gradually to reach a final concentration of 2.4 mM. The strips were then incubated in a CO_2 incubator at 37°C for 2 days in serum-free DMEM containing 5 mM Na_2ATP, 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 10 μg ml^{-1} plasmids.

Plasmids

pEGFP-N1 vectors encoding human wild-type α-actinin (full length, amino acids 1–892) and the rod domain

of α-actinin (53 kDa) (amino acids 250–892) were generously provided to us by Dr Carol Otey (University of North Carolina-Chapel Hill) and Dr Fredrick M. Pavalko (Indiana University School of Medicine). The EGFP is fused to the C-terminus of full-length α-actinin and the α-actinin rod domain (Laukaitis *et al.* 2001).

Cell dissociation and live cell imaging

Smooth muscle cells were enzymatically dissociated from tracheal muscle strips as previously described (Opazo Saez *et al.* 2004; Zhang *et al.* 2005). The localization of GFP-labelled α-actinin was monitored in live freshly dissociated cells plated onto glass coverslips by scanning them once per sec for 60 s using a Zeiss LSM 510 laser scanning confocal microscope with an Apo ×63 (NA 1.4) oil immersion objective. EGFP was excited with a 488 nm argon laser light, and fluorescence emissions were collected at 500–530 nm. The optical pinhole was set to resolve optical sections of ∼1 μm in cell thickness. Contraction was stimulated by adding ACh to the medium bathing the cell on the coverslip, to achieve a concentration of 100 μM.

Analysis of protein localization by immunofluorescence

The effects of ACh stimulation on the localization of endogenous and recombinant cytoskeletal proteins was evaluated as previously described (Opazo Saez *et al.* 2004; Zhang *et al.* 2005). The freshly dissociated smooth muscle cells were stimulated with 10^{-4} M ACh or left unstimulated, fixed and reacted with primary antibodies specific for the proteins of interest (α-actinin, talin, GFP-Rod or GFP-α-actinin, vinculin, paxillin) and with secondary antibodies (Alexa Fluor 488 and Alexa Fluor 546). The cellular localization of fluorescently labelled proteins were determined using a Zeiss LSM 510 laser scanning confocal microscope with an Apo ×63 oil-immersion objective (NA 1.4). Alexa 488-labelled (green) proteins were excited with a 488 nm argon laser light, and fluorescence emissions were collected at 500–530 nm. The fluorescence of Alexa 546-labelled (red) proteins was excited with a helium/neon laser at 543 nm, and emissions were collected at 565–615 nm. The optical pinhole was set to resolve optical sections of ∼1 μm in cell thickness.

Image analysis

Images of smooth muscle cells were analysed for regional differences in fluorescence intensity of labelled proteins by quantifying the pixel intensity with a series of six cross-sectional line scans along the entire length of each cell as previously described (Zhang *et al.* 2005) (Fig. 1*B*). The area of the nucleus, evident as a dark unstained area, was excluded from the analysis. The ratio of pixel intensity between the cell periphery and interior was determined for each line scan by calculating the ratio of the average maximum pixel intensity at the cell periphery to the minimum pixel intensity in the cell interior. The ratios of pixel intensities between the cell periphery and the cell interior for all of the six line scans performed on a given cell were averaged to obtain a single value for the ratio for each cell. The ratio of fluorescence intensity at the cell periphery to that at the cell interior was compared in cells at rest and in cells stimulated with ACh (10^{-4} M). For some cells, a series of optical sections from the bottom to the top of each cell was obtained at 0.6 μm intervals (see online Supplemental material, videos 1 and 2).

Immunoprecipitation of proteins

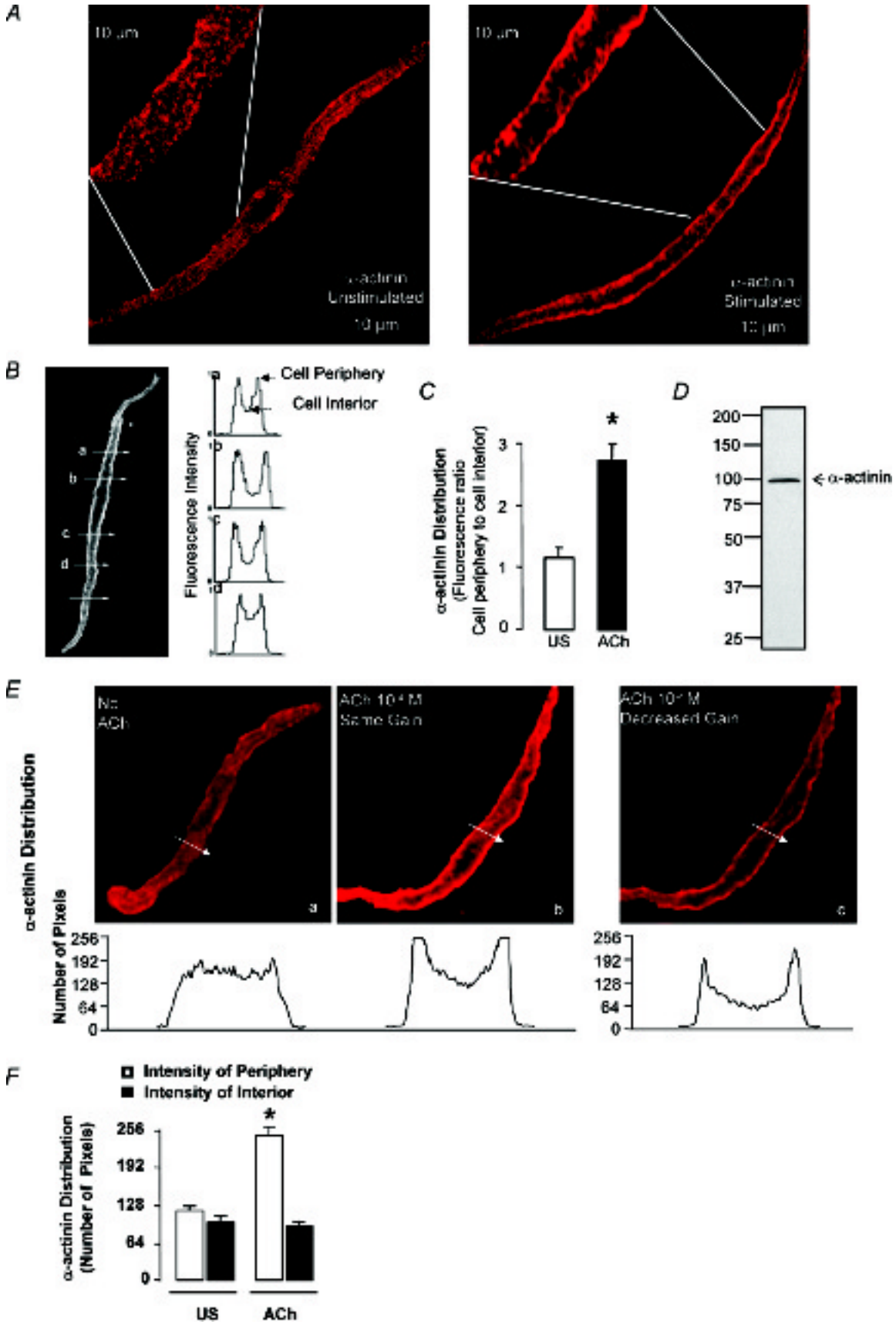
Pulverized muscle tissues were mixed with extraction buffer containing 1% Np-40, 20 mM Tris·HCl at pH 7.4, 0.3% NaCl, 10% glycerol, 2 mM EDTA, phosphatase inhibitors (mM: 2 sodium orthovanadate, 2 molybdate, and 2 sodium pyrophosphate), and protease inhibitors (mM: 2 benzamidine, 0.5 aprotinin, and 1 phenylmethylsulphonyl fluoride). Each sample was centrifuged at low speed (14 000 r.p.m., 16 000 g) for the collection of supernatant. Muscle extracts containing equal amounts of protein were precleared for 30 min with 50 μl of 10% protein A-sepharose and then were incubated overnight with monoclonal antibody against α-actinin to immunoprecipitate α-actinin. Samples were then incubated for 2 h with 125 μl of a 10% suspension of protein A-Sepharose beads conjugated to rabbit anti-mouse immunoglobulin. Immunocomplexes were washed four times in a buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Triton X-100. All procedures of immunoprecipitation were performed at 4°C.

Measurement of myosin light chain phosphorylation

Frozen muscle strips were immersed in dry ice precooled acetone containing 10% w/v trichloroacetic acid and 10 mM dithiothreitol. Proteins were extracted in 8 mM urea, 20 mM Tris base, 22 mM glycine and 10 mM dithiothreitol. Phosphorylated and unphosphorylated myosin lights chains were separated by glycerol-urea polyacrylamide gel electrophoresis, transferred to nitrocellulose then probed by western blotting (Zhang *et al.* 2005). The ratio of phosphorylated to unphosphorylated myosin light chains (MLCs) was determined by scanning densitometry.

Analysis of F-actin and G-actin

The relative proportions of F-actin and G-actin in smooth muscle tissues were analysed using an assay kit from Cytoskeleton (Denver, CO, USA) as previously described (Zhang *et al.* 2005). Briefly, each of the tracheal smooth muscle strips was homogenized in



200 μl of F-actin stabilization buffer (50 mM PIPES, pH 6.9, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 5% glycerol, 0.1% Triton X-100, 0.1% Nonidet P-40, 0.1% Tween-20, 0.1% β-mercaptoethanol, 0.001% antifoam, 1 mM ATP, 1 μg ml^{-1} pepstatin, 1 μg ml^{-1} leupeptin, 10 μg ml^{-1} benzamidine, and 500 μg ml^{-1} tosyl arginine methyl ester). Supernatants of the protein extracts were collected after centrifugation at 150 000 *g* for 60 min at 37°C. The pellets were resuspended in 200 μl of ice-cold distilled water containing 10 μM cytochalasin D and then incubated on ice for 1 h to depolymerize F-actin. The resuspended pellets were gently mixed every 15 min. Four microlitres of supernatant (G-actin) and pellet (F-actin) fractions were subjected to immunoblot analysis using antiactin antibody (clone AC-40; Sigma). The ratios of F-actin to G-actin were determined using densitometry.

Electron microscopy

Strips of muscle tissues were fixed at room temperature in 2% paraformaldehyde/2% glutaraldehyde in 0.075 M cacodylate containing 1.2 mM calcium and 4.5% sucrose at pH 7.4. Longitudinal sections 70–80 nm thick were then cut with a Leica UCT ultramicrotome (Lecia, Bannockbum, IL, USA) using a Diatome diamond knife (Diatome/Electron Microscopy Sciences, Fort Washington, PA, USA). The tissue sections were picked up on 200 mesh copper grids and stained with uranyl acetate and lead citrate. They were then viewed with a Tecnia G2 BioTwin electron microscope (FEI, Hillsboro, OR) at a magnification of ×49 000.

Results

α-Actinin is rapidly recruited to the cell membrane during contractile stimulation of smooth muscle with acetylcholine

We studied the effect of stimulation with acetylcholine (ACh) on the cellular localization of α-actinin in differentiated smooth muscle cells that were freshly dissociated from smooth muscle tissues. Small strips of trachealis muscle tissue were dissected from canine tracheas, thoroughly cleaned of connective and epithelial tissues, and enzymatically dissociated (Opazo Saez *et al.* 2004). After dissociation, cells were plated onto glass coverslips, stimulated with 10^{-4} M ACh or left unstimulated, fixed and labelled by immunofluorescence to determine the distribution of α-actinin (Fig. 1).

The localization of α-actinin in ACh-stimulated and unstimulated smooth muscle cells was evaluated by laser-scanning confocal microscopy. In unstimulated cells, α-actinin appeared in punctuate longitudinal arrays throughout the interior of the cell and along the cell exterior membrane (Fig. 1*A*), consistent with previous reports that α-actinin localizes in dense bodies in the cytoplasm and in dense plaques along the plasma membrane of differentiated smooth muscle cells (Geiger *et al.* 1981; Fay *et al.* 1983; Small, 1985). In cells that were stimulated with ACh, the fluorescence intensity of α-actinin along the membrane was increased relative to the interior of the cell (Fig. 1*A*).

The cellular distribution of α-actinin was analysed in unstimulated and stimulated smooth muscle cells obtained from tracheal tissues from 10 experiments. The distribution of α-actinin in each cell was quantified as previously described (Opazo Saez *et al.* 2004; Zhang *et al.* 2005) (Fig. 1*B*). In the unstimulated cells ($n = 68$), the ratio of the fluorescence intensity of α-actinin in the cell periphery to that in the cell interior was 1.3 ± 0.1 (Fig. 1*C*), indicating a slightly higher concentration of α-actinin at the membrane relative to the interior of the cell. In cells stimulated with ACh ($n = 48$), fluorescence intensity for α-actinin was 2.7 ± 0.1 times higher at the cell membrane than the cell interior, indicating that α-actinin is recruited to the membrane of smooth muscle cells in response to stimulation with ACh (Fig. 1*C*). When ACh was washed out of the cells, the fluorescence distribution

Figure 1. α-Actinin redistributes to the cell membrane in response to ACh stimulation
A, representative confocal images of fixed single freshly dissociated tracheal smooth muscle cells in which α-actinin is visualized by immunofluorescence. Left, unstimulated cell; right, ACh-stimulated cell. A section of each cell is shown at increased magnification in the upper left corner of each panel. *B*. Method for the analysis of cytoskeletal protein distribution within each cell. Each of six cross-sectional lines provided a fluorescence intensity profile that described protein distribution (*a–d*). The ratios of pixel intensity for each protein between the cell periphery and the cell interior was calculated from the average of the peak pixel intensities at the cell periphery to the average pixel intensity in the cell interior. The average ratio of all lines was calculated for each cell. *C*, mean ± S.E.M. of fluorescence intensity ratios for α-actinin in stimulated (ACh, $n = 48$) and unstimulated (US, $n = 68$) cells. *Significant difference between ratios. *D*, specificity of α-actinin is shown by an immunoblot of whole muscle extracts. *E*, raw values for pixel intensities across line scans of optical sections from an unstimulated cell (*a*) and a stimulated cell (*b*) that were immunofluorescently stained for α-actinin. Images (*a*) and (*b*) are shown at the same gain. Pixel intensity is similar in amplitude in the cell interior for both cells. In the stimulated cell, pixel intensity at the membrane is much higher than in the unstimulated cell and saturates the image. Therefore the same stimulated cell (*b*) is also illustrated at lower gain (*c*). Gain was sometimes reduced for images of stimulated cells to obtain greater resolution and to enable accurate calculation of fluorescence ratios. *F*, mean values for pixel intensity for stimulated and unstimulated cells ($n = 18$). *Significant difference between periphery and interior. Error bars are S.E.M.

rapidly returned to that observed in the unstimulated cells (data not shown). Figure 1*D* shows the specificity of the monoclonal α-actinin antibody in canine tracheal smooth muscle tissue extracts.

Raw values for pixel numbers across line scans of single optical sections obtained on stimulated and unstimulated cells that were immunostained for α-actinin are illustrated in Fig. 1*E* and *F*. The stimulated cell Fig 1*Eb* is also illustrated in Fig. 1*Ec*) at lower gain, because the increase in fluorescence intensity during stimulation causes saturation of the fluorescence signal at the membrane. This decreases resolution of the image and results in inaccurate quantification. In both stimulated and unstimulated cells, the fluorescence levels in the cytoplasm are well above background (Figs 1*E* and *F*). In the stimulated cells, the fluorescence intensity at the membrane is markedly higher than in unstimulated cells, whereas the mean fluorescence intensity observed in the cytoplasm is only slightly reduced, probably because the protein has a much larger volume of distribution in the cytoplasm than at the membrane.

The localization of α-actinin in smooth muscle cells was also evaluated by generating a series of optical sections of the cells in the Z plane (Supplemental material, videos 1 and 2). In the attached video images (Supplemental material, videos 1 and 2), a series of optical sections from the bottom to the top of the cells is shown for an unstimulated cell and an ACh-stimulated cell that were fixed and visualized by immunofluorescence. Throughout the interior sections of the ACh-stimulated cell, α-actinin fluorescence intensity is higher in the cell periphery than in the cell interior. In contrast, in the unstimulated cell, α-actinin fluorescence intensity is distributed more evenly throughout the cell interior and periphery. Thus the increased fluorescence at the cell periphery cannot be attributed to artifacts of optical sectioning.

To determine whether other contractile stimuli also elicit the translocation of α-actinin, we also tested the effects of 10^{-5} M histamine on the localization of endogenous α-actinin. The redistribution of α-actinin to the membrane was similar in response to stimulation with histamine and ACh (data not shown).

Time course of α-actinin relocalization during contractile stimulation

The time course of changes in the cellular distribution of α-actinin in response to stimulation with ACh was evaluated by expressing EGFP-tagged wild-type α-actinin (Laukaitis *et al.* 2001) in smooth muscle tissues using the technique of 'reversible permeabilization' (also called 'chemical loading') that we have previously described (see Methods). This procedure has no effect on the physiological properties of the muscle tissues (Tang *et al.* 2002, 2003; Tang & Gunst, 2004; Opazo Saez *et al.* 2004; Zhang *et al.* 2005). Cells were enzymatically dissociated from the tissues and the percentage of cells exhibiting EGFP fluorescence was determined by confocal fluorescence microscopy. Most of the dissociated cells (80–90%) exhibited EGFP fluorescence. No fluorescence was observed in cells from tissues not treated with plasmids (Fig. 2*A*).

Freshly dissociated smooth muscle cells expressing the recombinant EGFP–α-actinin were visualized live during stimulation with 10^{-4} M ACh. Cells were scanned once per second for 5 s before stimulation and for 30–60 s after stimulation with 10^{-4} M ACh (Fig. 2*B* and online Supplemental material, video 3). Before stimulation, EGFP fluorescence was observed throughout the interior of the cell. EGFP fluorescence intensity increased at the cell periphery and decreased in the cell interior within seconds of application of the ACh. Transient filopodial extensions were also observed during contractile stimulation of the cell (Fig. 2*B* and video 3). Similar results were observed in 20 cells dissociated from five different tissues (6–8 cells each tissue). Substantial shortening of cells could be obtained in response to stimulation with ACh; however, when there was significant movement it was difficult to monitor images from the same optical section over the entire time course of the contraction as the cell shortened and thickened. Furthermore, substantial shortening sometimes caused cells to detach from the glass coverslip. Therefore, to inhibit shortening, we allowed cells to develop adherence to the glass coverslip for approximately 1 h, which minimized changes in shape during stimulation.

Stimulation with ACh increases the association of α-actinin with β_1-integrin proteins

α-Actinin binds directly to β-integrin proteins *in vitro* (Otey *et al.* 1990), and can be coprecipitated with integrin proteins from fibroblasts (Edlund *et al.* 2001). To evaluate the effect of stimulation with ACh on the interaction of α-actinin with β-integrin proteins, α-actinin was immunoprecipitated from extracts of stimulated or unstimulated muscle tissues. Immunoblot analysis was used to evaluate the content of β_1-integrin and actin in the α-actinin immunocomplexes.

β_1-Integrin and actin were both found in α-actinin immunocomplexes precipitated from stimulated and unstimulated muscles (Fig. 3*A*). The ratio of β_1-integrin to α-actinin in the immunocomplexes from muscles stimulated with ACh increased significantly, suggesting that the α-actinin that localized at the membrane of the stimulated cells is associating with β-integrin complexes. In contrast, ACh stimulation did not have a significant effect on the amount of actin detected in the α-actinin immunocomplexes from stimulated and unstimulated muscles (Fig. 3*B*).

Expression of the rod domain of α-actinin inhibits the increase in association of β-integrin proteins with α-actinin in ACh-stimulated muscle tissues

The α-actinin rod domain contains a binding site for β-integrin but not actin, and has been shown to bind to the cytoplasmic tails of β-integrins *in vitro* (Otey *et al.* 1990). We expressed GFP fusion proteins for the α-actinin rod domain in tracheal smooth muscle tissues in order to block the association between endogenous α-actinin and the cytoplasmic tails of β_1-integrins (Otey *et al.* 1990; Pavalko & Burridge, 1991). We anticipated that they would bind to β-integrin complexes in the smooth muscle cells *in vivo*, and prevent endogenous α-actinin from being recruited to membrane-binding sites in response to stimulation with ACh, thus disrupting linkages between β-integrins and actin filaments.

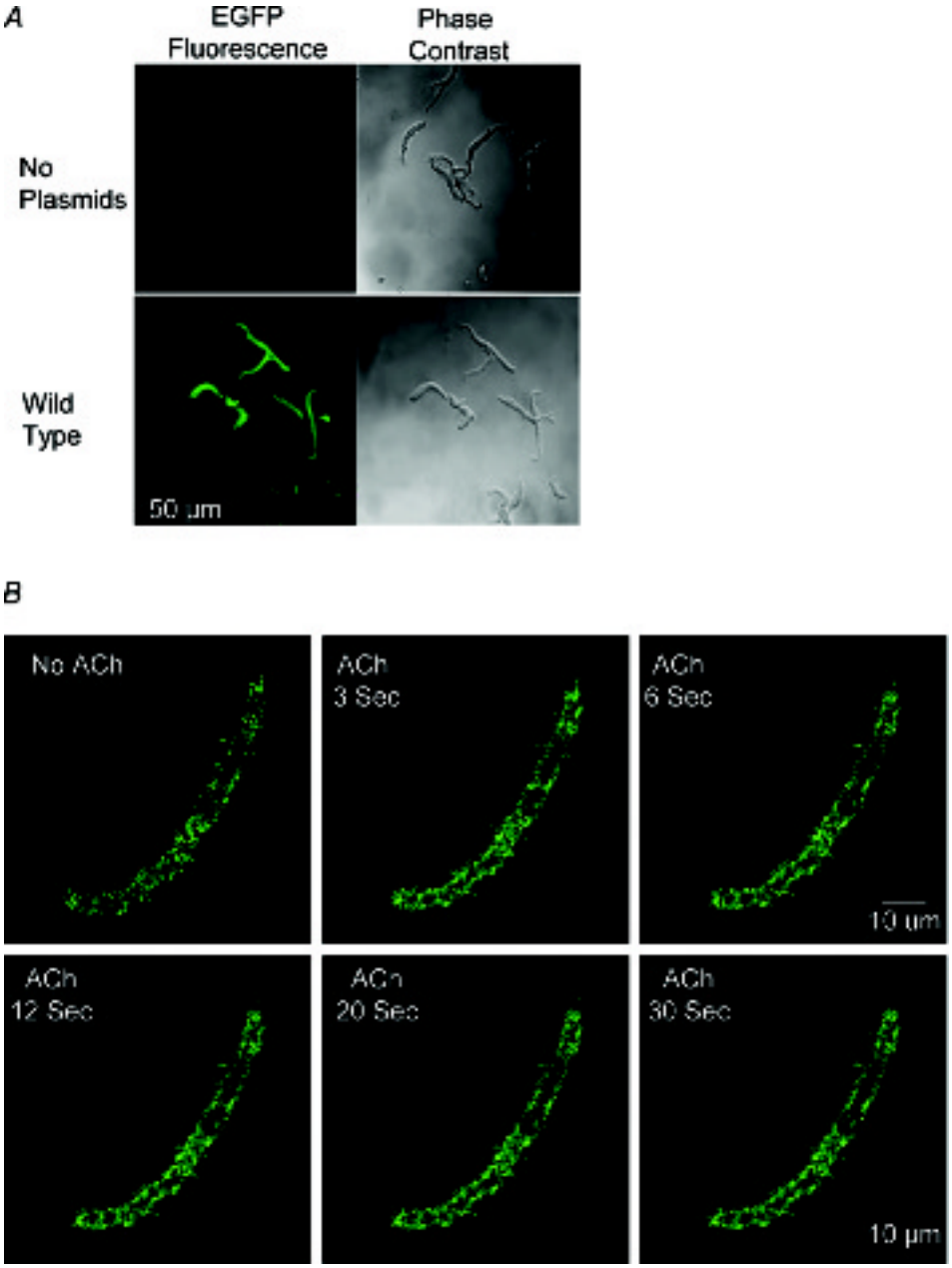


Figure 2. α-Actinin rapidly relocalizes to the cell membrane in response to contractile stimulation
A, EGFP fluorescence was detected in more than 80% of cells dissociated from the tissues transfected with EGFP–α-actinin plasmids. No cells dissociated from tissues that were not transfected with plasmids exhibited fluorescence. *B*, live cells freshly dissociated from tissues expressing EGFP–α-actinin were scanned once per second for 60 s during stimulation with ACh. GFP–α-actinin moved rapidly to the cell periphery in response to ACh. These images are excerpted from a real-time video available online as Supplemental material (Video 3).

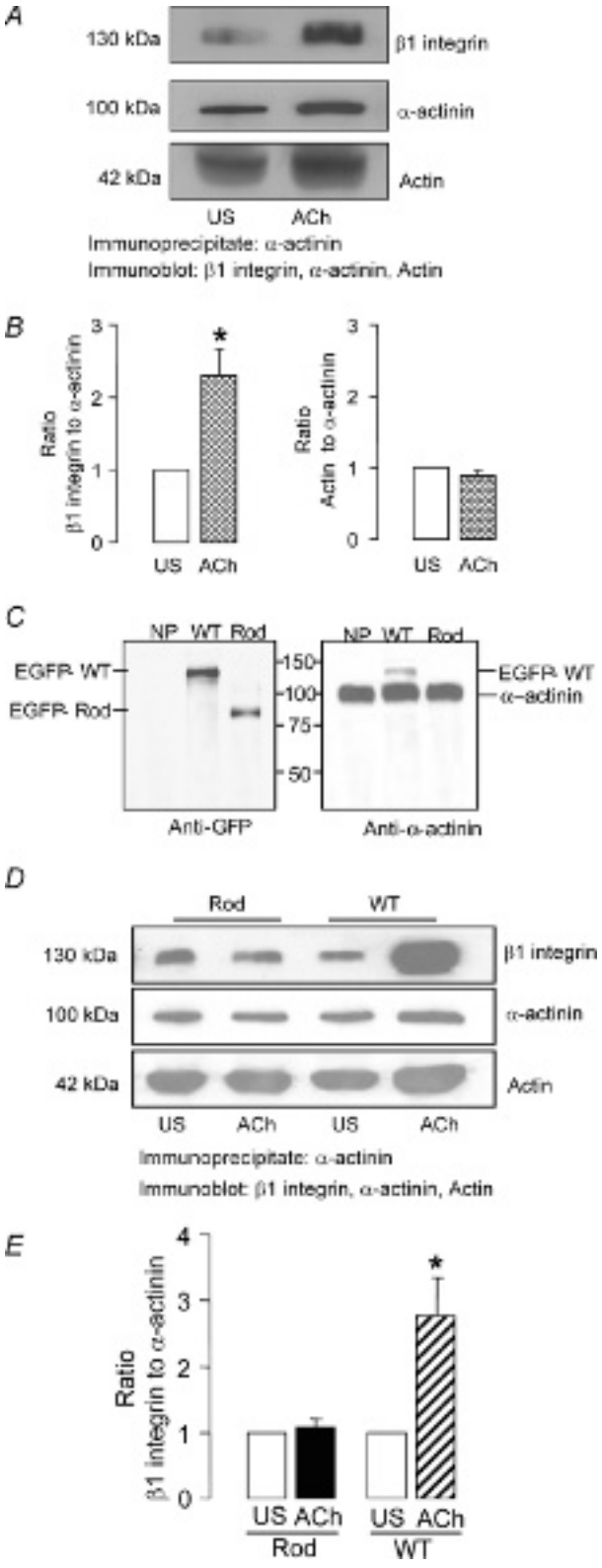


Figure 3. Expression of the rod domain of α-actinin inhibits the association of β_1-integrins with α-actinin in response to ACh
A, immunoblots of α-actinin immunoprecipitates from an unstimulated (US) and a stimulated (ACh) muscle. *B*, *significantly more β_1-integrin coprecipitated with α-actinin from homogenates of ACh-stimulated muscles than from homogenates of unstimulated tissues (US) ($n = 6$, $P < 0.05$). The amount of actin that coprecipitated with α-actinin from US and ACh tissues was not statistically different ($n = 5$). *C*, EGFP α-actinin (about 130 kDa) and EGFP-rod domain of α-actinin (about 80 kDa) are detected in immunoblots of extracts from tissues transfected with EGFP α-actinin (WT) and EGFP-rod domain (Rod) plasmids, but not in untreated tissues (NP). Antibody against α-actinin detects the 130 kDa GFP α-actinin WT protein but not the 80 KDa α-actinin rod domain (Rod). *D*, ACh stimulation increased the coprecipitation of β_1-integrin with α-actinin in tissues expressing EGFP wild-type (WT) α-actinin, but not in tissues expressing the EGFP-rod domain. *E*, *significant difference between mean ratios in US and ACh-stimulated muscle tissues ($P < 0.05$, $n = 6$). Error bars are S.E.M.

The effect of expression of the rod domain of α-actinin on the interaction of endogenous α-actinin with β-integrin proteins was evaluated in homogenates from muscles that were stimulated with ACh or unstimulated. The effects of expression of wild-type α-actinin were also evaluated to control for any possible effects of over-expression of the α-actinin protein. α-Actinin protein complexes were immunoprecipitated from muscle extracts using a monoclonal α-actinin antibody (Clone BM75.2, Sigma Co) that reacts with the full-length α-actinin protein but does not react with the recombinant α-actinin rod domain peptide (Fig. 3*C*).

In extracts from tissues expressing wild-type α-actinin, the amount of β-integrin that coimmunoprecipitated with α-actinin was significantly greater in tissues stimulated with ACh relative to that in unstimulated tissues, similar to results observed in tissues not treated with plasmids (Fig. 3*D* and *E*). In contrast, in α-actinin immunocomplexes obtained from muscle tissues expressing the rod domain of α-actinin, the amount of β_1-integrin protein that coprecipitated with α-actinin was similar in tissues stimulated with ACh compared to unstimulated muscles. Expression of the rod domain of α-actinin did not significantly affect the coprecipitation of actin with α-actinin in stimulated or unstimulated muscle tissues. These observations support our premise that the EGFP α-actinin rod domain proteins expressed in the muscle tissues bind to β-integrin proteins and inhibit the recruitment of endogenous α-actinin to integrin complexes during stimulation with ACh.

Expression of the rod domain of α-actinin inhibits the redistribution of α-actinin to the membrane in response to contractile stimulation of smooth muscle

We expressed GFP fusion proteins for the α-actinin rod domain in tracheal smooth muscle tissues to determine whether the association between the rod domain of α-actinin and the β-integrin complexes is necessary for the recruitment of α-actinin to the membrane in response to contractile stimulation. We hypothesized that the rod

domain peptides would bind to the cytoplasmic tails of β-integrins, and thereby prevent the recruitment of endogenous α-actinin to these sites.

We evaluated the localization of endogenous α-actinin in smooth muscle cells freshly dissociated from muscle strips transfected with the EGFP rod domain of α-actinin (Fig. 4*A*). Cells were fixed either unstimulated or after ACh stimulation, and double-stained with antibodies to α-actinin and GFP. Results were compared with cells from muscle tissues treated with plasmids for wild-type α-actinin.

In smooth muscle cells expressing the rod domain of α-actinin, GFP immunofluorescence was greater at the cell membrane in both stimulated and unstimulated cells (Fig. 4*A* and *B*). In contrast, the fluorescence signal from endogenous α-actinin was observed throughout the cytoplasm in both unstimulated cells and stimulated cells. The fluorescence intensity ratio for the GFP-tagged rod domain was 2–3 times higher at the cell periphery than the cell interior in both unstimulated ($n = 24$) and ACh-stimulated ($n = 32$) cells, whereas the fluorescence intensity ratio for α-actinin was slightly above 1 in both stimulated ($n = 32$) and unstimulated ($n = 24$) cells. We conclude that the α-actinin rod peptide localizes at the cell membrane in integrin complexes and inhibits the recruitment of endogenous α-actinin to the cell membrane in response to stimulation with ACh. In dissociated smooth muscle cells expressing EGFP–wild-type α-actinin, there were no differences in the distribution of proteins detected using antibodies to GFP and α-actinin (Fig. 4*C* and *D*). Only the recombinant EGFP α-actinin proteins are detected with the GFP antibody; whereas the α-actinin antibody reacts with both endogenous α-actinin and GFP wild-type α-actinin proteins. In unstimulated cells, the proteins stained by both antibodies were distributed throughout the cell, whereas in cells stimulated with ACh, proteins stained by both antibodies were more concentrated at the cell membrane relative to the cell interior (Fig. 4*C*). The ratios of fluorescence intensity for total α-actinin (2.6 ± 0.3) and for EGFP-tagged wild-type α-actinin (2.6 ± 0.4) in cells stimulated with ACh ($n = 28$) were not statistically different from each other, but were different from those in unstimulated cells (Fig. 4*D*, $n = 20$). This suggests that the expression of the recombinant wild-type α-actinin did not alter the localization of endogenous α-actinin.

Expression of the α-actinin rod domain does not inhibit the recruitment of paxillin, vinculin or talin to the membrane in response to ACh

We previously demonstrated that ACh stimulation induces redistribution of the cytoskeletal proteins paxillin, talin and vinculin to the cell membrane in freshly dissociated tracheal smooth muscle cells (Opazo Saez *et al.* 2004). To determine whether expression of the rod domain of α-actinin inhibits the recruitment of these proteins to the cell membrane in response to ACh, we evaluated the localization of paxillin, vinculin and talin in smooth muscle cells freshly dissociated from muscle strips expressing the EGFP rod domain of α-actinin. Cells were double-stained with antibodies to α-actinin and vinculin (Fig. 5*A* and *B*), talin and GFP (Fig. 5*C* and *D*), or paxillin and GFP (Fig. 5*E* and *F*). Although the recruitment of endogenous α-actinin was inhibited by expression of the rod domain á-actinin, vinculin redistributed to the cell membrane in response to ACh stimulation (Fig. 5*A* and *B*). Similarly, talin and paxillin were recruited to the membrane of cells expressing the GFP rod domain of α-actinin in response to ACh (Fig. 5*C*–*F*).

Expression of the rod domain of α-actinin inhibits tension development in smooth muscle tissues

We determined the effect of expression of the α-actinin rod domain fusion proteins on tension development in response to stimulation with ACh in intact smooth muscle tissue strips (Fig. 6). In smooth muscle strips expressing the rod domain of α-actinin, isometric contractile force in response to 5 min stimulation with ACh was significantly reduced to $35.7 \pm 7.4\%$ of that in untreated smooth muscle tissues ($n = 42$, $P < 0.05$). In contrast, in tissues expressing wild-type α-actinin, EGFP, or tissues not treated with plasmids, isometric force in response to stimulation with ACh was not significantly different from the pre-incubation force. There were no significant differences in tension among the four groups of tissues before incubation. These observations suggest that the recruitment of α-actinin to integrin complexes is necessary for tension development in smooth muscle tissues.

Expression of the rod domain of α-actinin does not affect MLC phosphorylation in smooth muscle tissues

Phosphorylation of the 20 kDa light chain of myosin is widely recognized as a major cellular event in the initiation of cross-bridge cycling and smooth muscle contraction (Kamm & Stull, 1989). We evaluated the possibility that recruitment of the rod domain of α-actinin to integrin complexes might affect tension development by disrupting signalling pathways that regulate the activation of myosin. The effects of stimulation with ACh on myosin light chain phosphorylation were compared in muscle tissues transfected with the rod domain of α-actinin (Rod), plasmids encoding wild-type α-actinin (WT), and tissues incubated with no plasmids (NP) (Fig. 7*A*). There were no significant differences in MLC phosphorylation in unstimulated or ACh-stimulated muscles expressing the rod domain of α-actinin, wild-type α-actinin or muscles

not treated with plasmids ($n = 8$, $P > 0.05$). Thus, the inhibition of contraction by the rod domain of α-actinin did not affect signalling pathways that regulate MLC phosphorylation (Fig. 7*B*).

Expression of wild-type or the rod domain of α-actinin does not affect actin polymerization in smooth muscle tissues

α-Actinin plays an important structural role in determining actin filament organization in both smooth

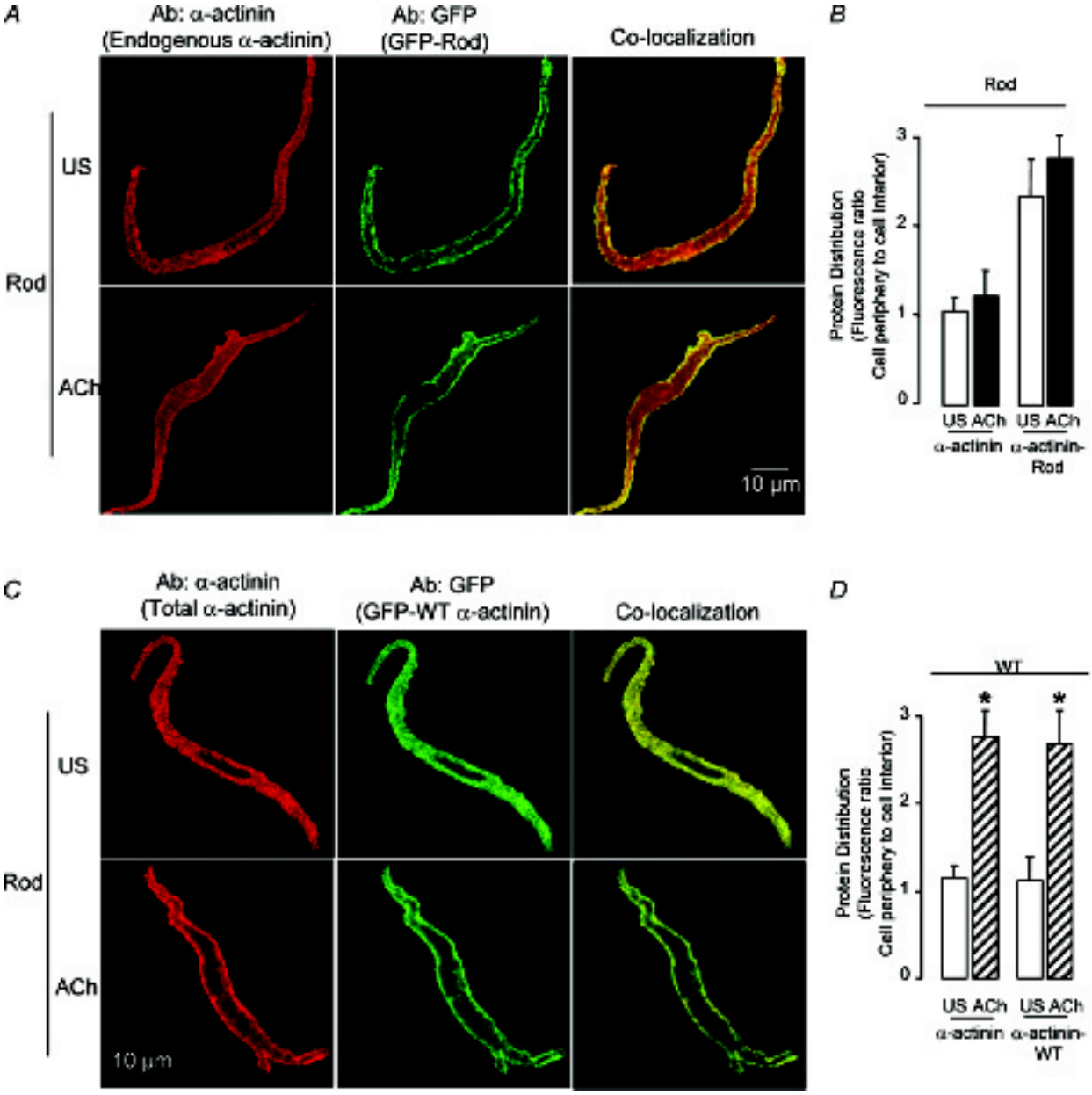
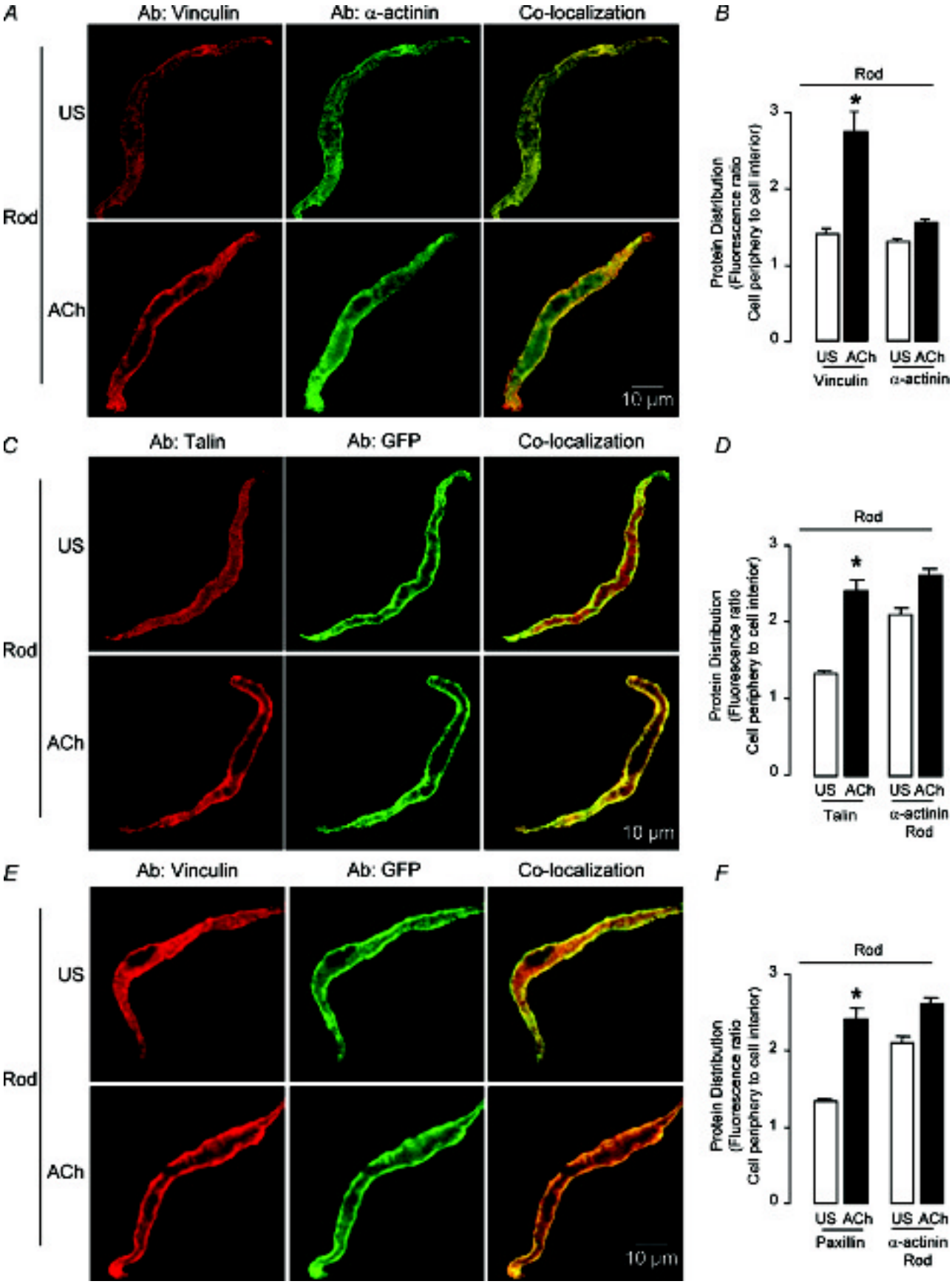


Figure 4. Expression of the rod domain of α-actinin inhibits the redistribution of endogenous α-actinin to the membrane in response to ACh stimulation

Cells were double-immunostained for EGFP and α-actinin, to assess the localization of recombinant EGFP-tagged α-actinin proteins and α-actinin. *A*, in tissues expressing the α-actinin rod domain (Rod), EGFP α-actinin–Rod immunofluorescence remained at the cell periphery while endogenous α-actinin was distributed throughout the cell in both unstimulated (US) and ACh-stimulated cells. *B*, ratios for EGFP-rod-domain proteins and endogenous α-actinin in cells from rod-domain-treated tissues. *Difference in ratios in ACh ($n = 32$) and US ($n = 24$) cells were not significant ($P > 0.05$). *C*, in cells from tissues expressing wild-type α-actinin (WT), the distributions of EGFP–WT-α-actinin and α-actinin fluorescence were similar. *D*, mean values of ratios for EGFP–WT α-actinin protein and α-actinin in cells expressing WT α-actinin. *Significant difference in ratio between ACh ($n = 28$) and US ($n = 20$) cells ($P < 0.05$). Error bars are S.E.M.



muscle and non-muscle cell types (Otey & Carpen, 2004). In cultured fibroblasts, the microinjection of proteolytic fragments of α-actinin, either the rod domain or the 27 KDa actin-binding domain, leads to the dissolution of stress fibres and focal adhesion complexes within 1–2 h, if the concentration of the injected fragments is sufficiently high ($>5\,\mu$M) (Pavalko & Burridge, 1991). In tracheal smooth muscle tissues, at least 70–80% of the actin is in a polymerized state in unstimulated tissues, and contractile stimulation induces a further increase in the amount of polymerized actin (Tang & Gunst, 2004; Zhang *et al.* 2005). In the present study, we used a cell fractionation assay (Cytoskeleton, Inc.) to analyse the proportions of G-actin and F-actin in extracts from unstimulated and stimulated muscle tissues expressing the rod domain of α-actinin (Tang & Gunst, 2004; Zhang *et al.* 2005) (Fig. 8*A*).

No significant differences were observed in the amounts of G- or F-actin among the strips expressing the rod domain of α-actinin, wild-type α-actinin or in strips not treated with plasmids (Fig. 8*B*). Thus, expression of the α-actinin proteins did not result in significant depolymerization of the actin cytoskeleton. Stimulation with ACh increased the ratio of F-actin to G-actin similarly in all three groups of smooth muscle tissues, even though in the rod-domain-treated tissues, force was significantly depressed to $37 \pm 6.1\%$ of force in untreated muscle tissues or tissues treated with wild-type α-actinin (data not shown).

The effect of expression of recombinant α-actinin proteins on the ultrastructure of smooth muscle tissues

We examined sections of tracheal smooth muscle tissues by electron microscopy to determine whether expression of the rod domain of α-actinin or wild-type α-actinin altered the ultrastructure or the cytoskeletal organization of the muscle cells (Fig. 9). After transfection with plasmids and 2 days' incubation, tissues were tested to confirm the inhibition of force by the α-actinin rod domain plasmids, and then fixed for electron microscopy. No differences in cytoskeletal organization or ultrastructure were evident in electron micrographs taken from muscle tissues expressing the rod domain of α-actinin, wild-type α-actinin, or untreated tissues. Although it is possible that more subtle structural alterations are present that are not apparent in electron micrographs, our results suggest that the inhibition of tension development caused by expression of the rod domain of α-actinin cannot be attributed to the disruption of cytoskeletal organization or cell structure.

Discussion

The widely accepted basis for active tension development during smooth muscle contraction is based on a paradigm in which the cytoskeletal organization of the smooth muscle cell remains fixed, and the sliding of actin and myosin filaments against each other is powered by the cyclical ATP-dependent cycling of actomyosin crossbridges leading to the generation of tension. Although there is little doubt that actomyosin crossbridge cycling is the fundamental mechanism for tension generation in smooth muscle, recent data have suggested that the structural organization of the cytoskeleton of differentiated smooth muscle cells and tissues may be far more labile than previously assumed (Gunst & Fredberg, 2003; Gunst *et al.* 2003; Kim *et al.* 2004; Herrera *et al.* 2004; Opazo Saez *et al.* 2004; Zhang *et al.* 2005) Studies of a number of smooth muscle tissue types have provided evidence that actin undergoes polymerization under various conditions of contraction in smooth muscle tissues, and that the polymerization of actin may be an essential step in tension generation (Adler *et al.* 1983; Saito *et al.* 1996; Jones *et al.* 1999; Mehta & Gunst, 1999; Barany *et al.* 2001; An *et al.* 2002; Cipolla *et al.* 2002; Herrera *et al.* 2004; Tang & Gunst, 2004; Flavahan *et al.* 2005; Zhang *et al.* 2005). Furthermore, there is evidence that the localization of structural proteins within adhesion sites may be dynamically regulated in response to the pharmacological stimulation of smooth muscle (Kim *et al.* 2004; Opazo Saez *et al.* 2004), although this remains controversial (Eddinger *et al.* 2005). A dynamic cytoskeletal organization

Figure 5. Expression of the rod domain of α-actinin does not inhibit the redistribution of vinculin, talin or paxillin to the membrane in response to ACh stimulation
Cells were dissociated from smooth muscle strips treated with plasmids for the rod domain of α-actinin and stimulated with 10^{-4} M ACh or left unstimulated (US). *A*, cells were double-stained for vinculin and α-actinin to assess the colocalization of vinculin and endogenous α-actinin. *B*, *significant difference between mean ratios for ACh ($n = 15$) and US ($n = 9$) cells from rod-domain-treated tissues ($P < 0.05$). *C*, cells were double-stained for EGFP and talin to assess the colocalization of GFP α-actinin rod domain and talin. *D*, mean values of fluorescence intensity ratios for talin and EGFP–α-actinin rod domain in cells from rod-domain-treated tissues. *Significant difference between mean ratios for ACh ($n = 12$) and US ($n = 8$) cells ($P < 0.05$). *E*, cells were double-stained for EGFP and paxillin to assess the colocalization of GFP α-actinin rod domain and paxillin. *F*, mean values of fluorescence intensity ratios for paxillin and EGFP–α-actinin rod domain in cells from rod-domain-treated tissues. *Significant difference between mean ratios for ACh ($n = 12$) and US ($n = 14$) cells ($P < 0.05$). Error bars are S.E.M.

may enable smooth muscle cells to adapt their shape to accommodate to external forces imposed on them under physiological conditions. The recruitment of cytoskeletal proteins to the sites of membrane adhesion may be necessary to strengthen connections between actin filaments and extracellular matrix that support the transmission of force generated by the contractile apparatus.

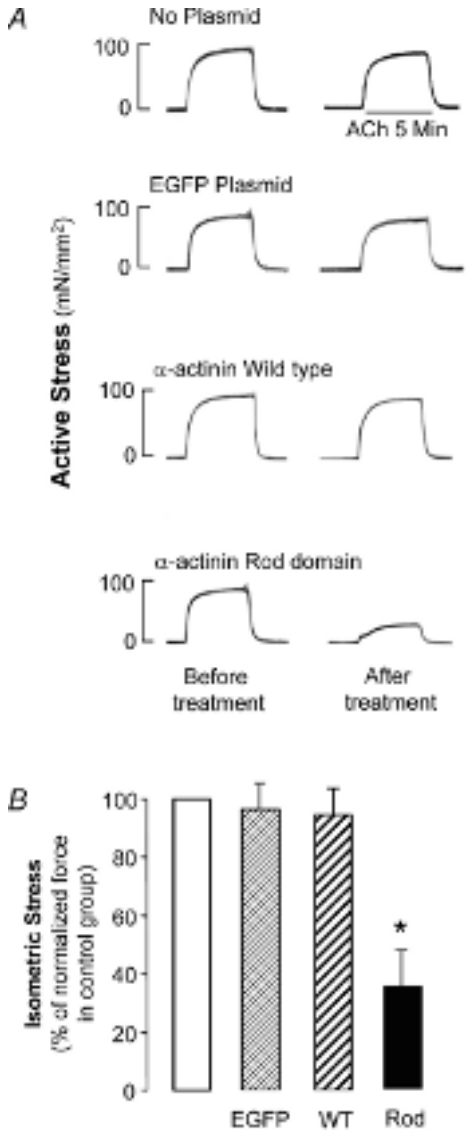


Figure 6. Expression of the rod domain of α-actinin in smooth muscle tissues inhibits active tension development
A, representative tracings from a single experiment showing contractile force in response to 10^{-5} M ACh in four muscle tissues before and after 2 days' incubation with no plasmids, EGFP plasmids, plasmids encoding wild-type α-actinin, or plasmids encoding the rod domain of α-actinin. *B*, mean isometric stress in smooth muscle tissues treated with plasmids encoding the rod domain of α-actinin (Rod), wild-type α-actinin (WT), EGFP, or with no plasmids (NP). Active stress was quantified as percentage of the normalized stress in the NP group. Values are means ± S.E.M. *Stress significantly different from NP group ($n = 42$, $P < 0.05$).

In the present study we provide several lines of evidence that α-actinin is recruited to integrin complexes in smooth muscle tissues and cells during stimulation with ACh. First, we show by immunofluorescence analysis that more α-actinin is at the membrane of freshly dissociated smooth muscle cells stimulated with ACh than in unstimulated cells. Second, we observe that GFP-labelled recombinant α-actinin expressed in smooth muscle tissues is recruited to the membrane of live freshly dissociated cells within seconds of their stimulation with ACh. Third, we find more β-integrin is associated with α-actinin immunocomplexes in precipitates from extracts of stimulated muscle tissues than in α-actinin immunocomplexes from

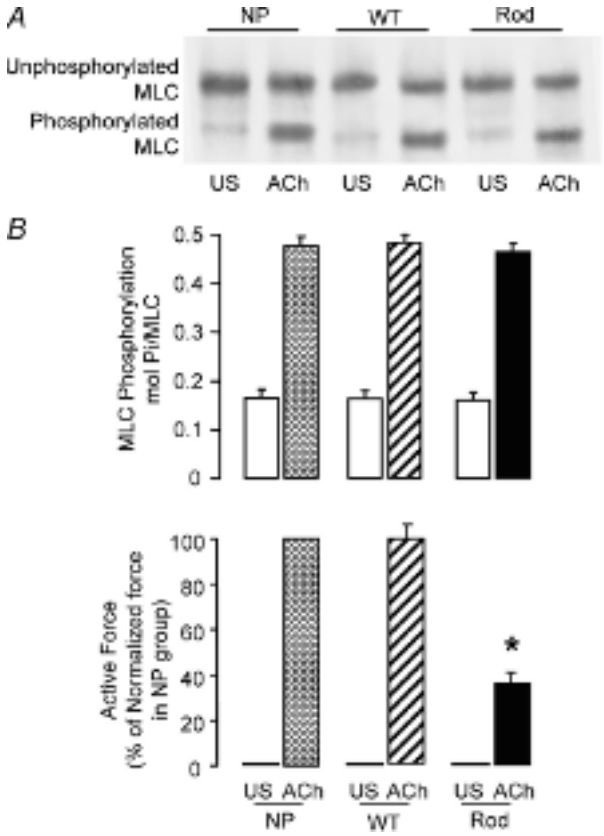


Figure 7. Expression of Rod domain of α-actinin does not inhibit the increase in myosin light chain (MLC) phosphorylation in response to ACh
Muscle tissues treated with plasmids encoding the rod domain of α-actinin (Rod), wild-type α-actinin (WT) or not treated with plasmids (NP) were stimulated with 10^{-4} M ACh for 5 min and then frozen for the measurement of MLC phosphorylation. *A*, representative immunoblot from a single experiment showing MLC phosphorylation in response to 10^{-4} M ACh in six muscle tissues treated with no plasmids (NP), plasmids encoding wild-type α-actinin (WT), or plasmids encoding the rod domain of α-actinin (Rod). *B*, active stress was significantly depressed in muscle tissues treated with plasmids encoding the rod domain of α-actinin; however, no significant differences were observed in MLC phosphorylation ($P > 0.05$, $n = 8$). Values shown are means ± S.E.M. *Significantly different than NP group.

unstimulated muscle tissues. These results demonstrate that the structure of adhesion sites in smooth muscle tissues is labile and dynamic, and that the recruitment of α-actinin to the membrane is regulated during contractile stimulation.

In smooth muscle cells, α-actinin is localized in dense bodies in the cytoplasm and at dense plaques at the cell membrane (Geiger *et al.* 1981; Fay *et al.* 1983; Small, 1985; Draeger *et al.* 1990). Studies of α-actinin in focal adhesions and stress fibres in fibroblasts have shown that α-actinin exchanges rapidly in both structures (Edlund *et al.* 2001; Peterson *et al.* 2004) Peterson *et al.* 2004 reported an increase in focal adhesion area occurring over a period of 30 min in contracting Swiss3T3 fibroblasts, and also observed a redistribution of α-actinin among fluorescent bands along stress fibres within these cells, with some areas of the cell losing α-actinin, and other areas gaining α-actinin. Fultz *et al.* 2000 reported that the stimulation of smooth muscle cells from the A7r5 aortic cell line with phorbol dibutyrate caused the highly granular filamentous organization of α-actinin in unstimulated cells to undergo remodelling within 10–30 min, to form peripheral bodies near the cell membrane that are intensely fluorescent for α-actinin.

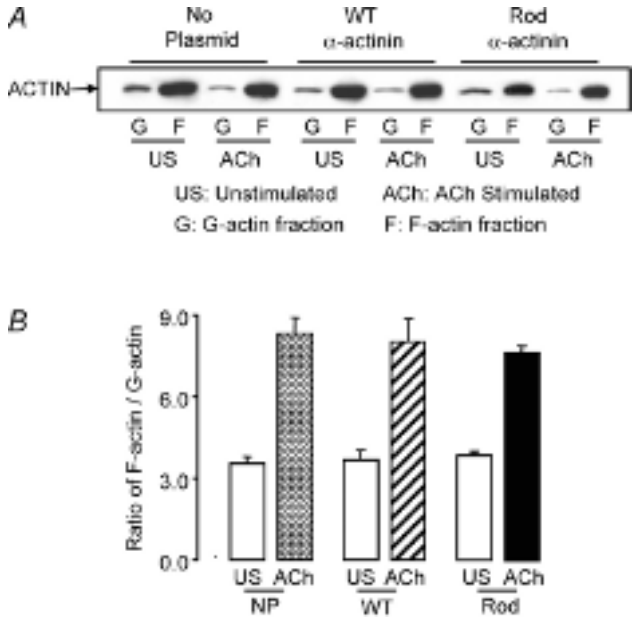


Figure 8. Expression of the rod-domain of α-actinin does not inhibit actin polymerization in muscle tissues in response to ACh stimulation
A, representative immunoblot of actin in soluble G-actin fraction (G) and insoluble F-actin fraction obtained from unstimulated (US) and ACh-stimulated (ACh) muscle tissues treated with Rod domain, WT α-actinin, or not treated with plasmids. *B*, mean $\pm$ S.E.M. of ratios of F/G-actin in unstimulated and ACh-stimulated muscle tissues from untreated (NP), Rod, and WT muscle tissues. Ratios of F/G-actin among the three groups were not significantly different among US or ACh groups of muscle tissues ($n = 6$, $P > 0.05$). Error bars are S.E.M.

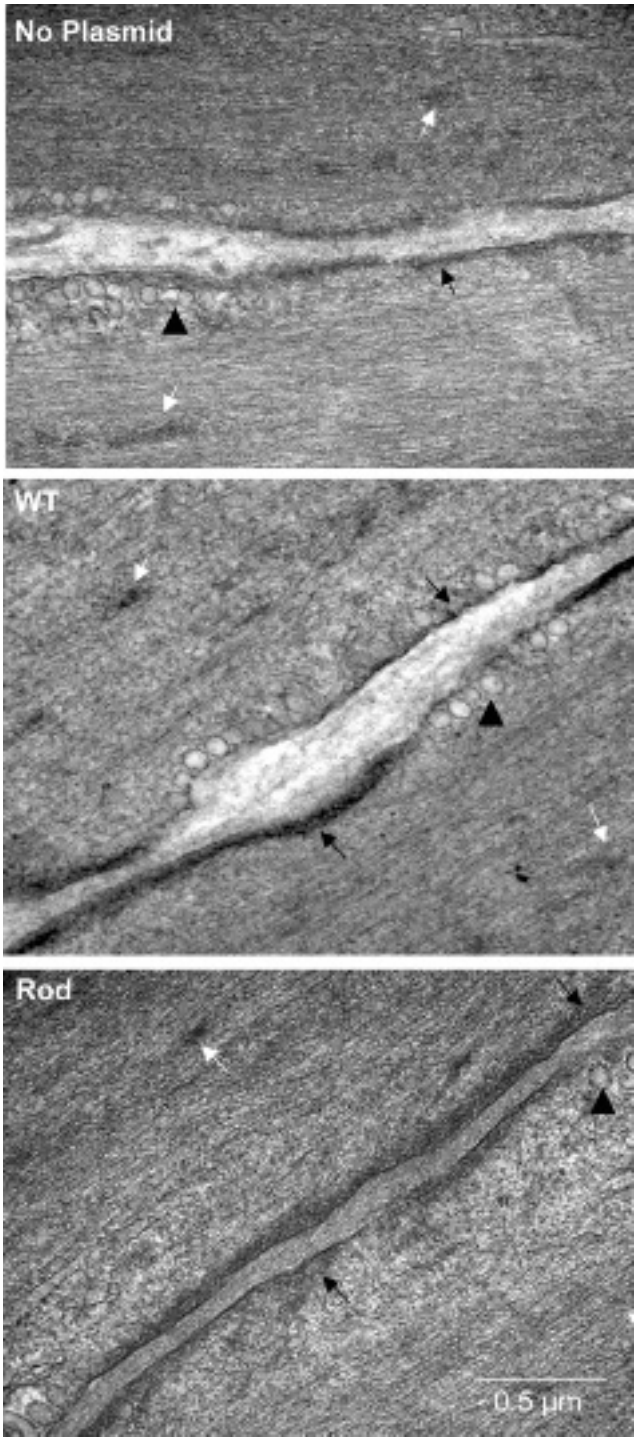


Figure 9. Effects of expression of wild-type (WT) α-actinin, rod-domain of α-actinin (Rod) or no plasmid treatment (NP) on the ultrastructure of smooth muscle tissues
Representative electron micrographs of 60 nm-thick longitudinal sections of unstimulated tracheal muscle tissues. No differences in structure or cytoskeletal organization were detected from electron micrographs of tissues expressing the rod domain of α-actinin (Rod), wild-type α-actinin (WT) or no plasmids. Black arrows point to membrane-associated dense plaques. White arrows point to cytoplasmic dense bodies. ▲ caveolae. Scale bar, 0.5 μm.

Our results suggest that α-actinin rapidly relocalizes to the membrane from other locations within the smooth muscle cell after contractile stimulation, but the source of the α-actinin that is recruited to the membrane is unclear. α-Actinin may be partially depleted from cytoplasmic cytoskeletal structures, or it may have a diffuse distribution in unstimulated cells and become more focally localized after stimulation. The mechanism by which α-actinin is recruited to the cell membrane is also unknown. Recently, Fraley *et al.* (2003, 2005) demonstrated that in cultured fibroblasts, the association/dissociation rate of α-actinin binding to actin filaments and integrin adhesion receptors is regulated by phosphoinositides, and that the dynamics of α-actinin is important for PI_3-kinase-induced reorganization of the actin cytoskeleton. Phosphoinositide binding inhibited the bundling activity of α-actinin by blocking the interaction of the actin-binding domain with actin filaments. During ACh stimulation of smooth muscle, the binding of phosphoinositides to α-actinin may regulate the recruitment of α-actinin to the cell membrane, and this recruitment may be important for actin remodelling.

Our results also suggest that the recruitment of α-actinin to adhesion sites at the smooth muscle membrane and its association with β-integrins is a necessary step in the process of active tension generation. The expression of an α-actinin peptide consisting of the integrin-binding rod domain of α-actinin in smooth muscle tissues markedly inhibited active tension generation in response to cholinergic stimulation. We also found that expression of this peptide blocked localization of endogenous α-actinin to the membrane in response to stimulation with ACh and inhibited the association between α-actinin and β-integrins. These results are consistent with the hypothesis that the α-actinin rod domain inhibits force generation by blocking the recruitment of endogenous α-actinin to the membrane and preventing its interaction with β-integrin.

The activation of actomyosin crossbridges is well established as the principal mechanism for the regulation of tension development in smooth muscle (Kamm & Stull, 1989). We therefore evaluated the possibility that the recruitment of α-actinin to integrin complexes affects the regulation of signalling pathways that lead to crossbridge activation. We found no decrease in MLC phosphorylation in muscle tissues expressing the rod domain of α-actinin, even though force was markedly depressed in these tissues. Thus, the inhibition of the recruitment of α-actinin to adhesion complexes in smooth muscle does not affect pathways that regulate the activation of myosin.

In cultured fibroblasts, the microinjection of high levels of α-actinin proteolytic fragments (approximately equal to or greater than that of endogenous α-actinin) results in a loss of both stress fibres and focal adhesions after 0.5–2 h, leading to cytoskeletal disorganization (Pavalko & Burridge, 1991). Expression of the rod domain of α-actinin might therefore inhibit tension development by causing disorganization of the cytoskeletal structure of the smooth muscle cells, or by preventing actin polymerization. In airway smooth muscle, the inhibition of actin polymerization inhibits the development of active tension (Mehta & Gunst, 1999; Zhang *et al.* 2005). However, in the present study, the α-actinin rod domain protein did not significantly alter the balance of G- and F-actin in smooth muscle tissues, either when they were unstimulated or after contractile stimulation with ACh. Furthermore, we did not observe significant effects of the α-actinin rod domain on the association of α-actinin with actin in α-actinin immunoprecipitates from unstimulated or stimulated tissue homogenates. We also examined by electron microscopy the ultrastructure of smooth muscle tissues expressing the rod domain of α-actinin. There was no evidence for disruption of the cytoskeletal organization or cellular morphology in tissues expressing the α-actinin rod domain peptides. The amount of recombinant α-actinin expressed in the smooth muscle tissues was substantially less than the amount of the endogenous α-actinin protein; thus the levels of rod domain peptides were unlikely to be high enough to elicit the disruption of the actin cytoskeleton that occurred when proteolytic fragments of α-actinin were microinjected into cells (Pavalko & Burridge, 1991).

α-Actinin plays a critical role in the dynamic formation of adhesion complexes during cell adhesion and crawling (Edlund *et al.* 2001). In cultured fibroblasts, talin, paxillin and vinculin are involved in the early stages of focal adhesion formation, and α-actinin is recruited to adhesion sites later to strengthen or stabilize integrin–cytoskeletal linkages (von Wichert *et al.* 2003). Ultrastructural studies have also documented the presence of α-actinin at membrane adhesion junctions of smooth muscle cells and tissues (Geiger *et al.* 1981; Fay *et al.* 1983; Small, 1985; Draeger *et al.* 1989, 1990). Thus, it is possible that the assembly of new linkages between the actin cytoskeleton and integrin proteins and/or the strengthening of existing integrin–cytoskeletal linkages may be a necessary step in the development of active tension during the contractile stimulation of smooth muscle, and α-actinin may play a critical role in this process.

In migrating cells, new adhesive complexes form at the leading edge of the cell to provide the traction necessary to move the cell body forward. These adhesive complexes have been proposed to form around a small cluster of ligand-bound integrins by the stepwise addition of structural and signalling proteins in temporal succession, which act to strengthen integrin–cytoskeletal connections (Laukaitis *et al.* 2001; Rajfur *et al.* 2002). In migrating fibroblasts, recent evidence suggests that the scaffolding protein paxillin is recruited to adhesion sites early in the process; whereas α-actinin subsequently colocalizes with

paxillin (Laukaitis *et al.* 2001). We previously reported that the paxillin and vinculin are recruited to the membrane of smooth muscle cells in response to contractile stimulation in differentiated freshly dissociated smooth muscle cells, and that the recruitment of vinculin was dependent on the recruitment of paxillin (Opazo Saez *et al.* 2004). In the present study, we found that paxillin, talin and vinculin were still recruited to the membrane of smooth muscle cells in response to stimulation by ACh when the recruitment of α-actinin was inhibited by expression of the α-actinin rod domain. Thus, our studies are consistent with observations in migrating fibroblasts, in that the recruitment of paxillin, talin and vinculin to the cell membrane during contractile stimulation was independent of the recruitment of α-actinin.

Evidence from several studies suggests that α-actinin reinforces integrin–cytoskeletal connections to enable them to withstand the force transmitted to the extracellular matrix during cell migration (Balaban *et al.* 2001; Bershadsky *et al.* 2003; von Wichert *et al.* 2003). The localization of α-actinin to the newly formed focal complexes is correlated with their ability to generate contractile force. Similarly, during the active contraction of differentiated smooth muscle, contractile stimulation may initiate the recruitment of α-actinin to strengthen integrin–cytoskeletal binding sites so that the force generated by actomyosin crossbridges can be transmitted to the extracellular matrix. Thus, expression of the rod domain of α-actinin may impair the assembly of adhesion sites and inhibit force by weakening integrin–cytoskeletal connections.

Our results provide further evidence that tension generation in smooth muscle is a complex event involving dynamic cytoskeletal processes that occur concurrently with the activation of crossbridge cycling. Although dynamic cytoskeletal events that occur during the initiation of contraction may mediate multiple functions, our observations are consistent with the possibility that the recruitment of cytoskeletal proteins to the sites of integrin-mediated cell adhesion may strengthen or reinforce linkages between actin filaments and integrin proteins, to support the transmission of contractile force to the extracellular matrix. The dynamism of the cytoskeleton during the process of contractile activation may enable smooth muscle cells within hollow organs to modulate their cytoskeletal structure to adapt to external forces imposed on them by external physiological events; this may enable the tissue to alter its contractility and mechanical properties to accommodate to diverse physiological conditions.

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Supplemental material

The online version of this paper can be accessed at:
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and contains supplemental material consisting of three videos. They are shown at 1 frame s^{-1} in QuickTime movie format.

Video 1. A series of optical sections from unstimulated smooth muscle cells fluorescently labelled for endogenous α-actinin.

Video 2. A series of optical sections from ACh-stimulated smooth muscle cells fluorescently labelled for endogenous α-actinin.

Video 3. The redistribution of GFP–α-actinin in a single freshly dissociated smooth muscle cell during stimulation with ACh.

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